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Inhibitory Effect of Histone on the Peroxidase Activity of the Hp/Hb Complex

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Summary: The human leukocyte nuclear histones can bind to haptoglobin (Hp), and thus interfere with subsequent binding of Hb. The Hp/Hb complex possesses peroxidase activity, but a Hp/histone complex does not. The inhibitory effect of histone molecules depends directly on the Hp and Hb, as well as the histone concentrations. The biological significance of the complex Hp/Hb as a measure of intravascular hemolysis and the interference of histones in this assay is briefly discussed.

Hemmungseffekt von Histonen auf die Peroxidase-Aktivität des Hp/Hb-Komplexes

Zusammenfassung: Histone der Kerne menschlicher Leukocyten können Haptoglobin (Hp) binden und die weitere Bindung an Hb verhindern. Der Hp/Hb-Komplex weist Peroxidase-Aktivitäten auf, die der Hp-Histon-Komplex nicht besitzt. Der Hemmeffekt der Histonmoleküle ist direkt von den Hp-, Hb- und Histon-Konzentrationen abhängig. Die biologische Bedeutung des Hp/Hb-Komplexes als Maß für die intravasale Hämolyse und die Beeinflussung dieses Nachweisversuchs durch Histone wird diskutiert.

Introduction

It was demonstrated some time ago that haptoglobin (Hp), a group of acidic proteins, has the ability to bind hemoglobin (Hb), and that this Hp/Hb complex, like the Hb or Hp separately, possesses a high peroxidase activity (1, 2). The peroxidase activity of the Hp/Hb complex depends on different factors and conditions, which were discussed previously in detail (8).

The binding mechanism of Hp to Hb is an electrostatic effect arising from interactions between the positively charged basic Hb and the negatively charged acidic Hp molecules (6). The biological significance of this effect is, of course, that the peroxidase activity of Hp, as well as Hb, is smaller than that of the Hp/Hb complex, and that the formation of the Hp/Hb complex is a measure of intravascular hemolysis.

However, this principle was of value providing it was valid to assume that other biological substances, such as histone, could not hinder the binding of Hb to Hp. Modifications of the structure of the molecules or changes in the conditions of the binding are accompanied, evidently in vitro, by alterations of the biological functions of the corresponding molecules.

The human leukocyte nuclei histones, a family of five different positively charged basic proteins, are bound in vivo to the chromosomal deoxyribonucleic acid (DNA), following the same molecular mechanism of binding as the Hp/Hb complex. The free histones can interact with other molecules (3) and play an important role in various other biological functions.

Thus free histone can cause alterations in the binding ability of the Hp to Hb and, of course, thereby affect the peroxidase activity of the Hp/Hb complex.

In the present paper, we report that the human leukocyte nuclear histones can bind to Hp and thus interfere with subsequent binding of Hb, and that Hp/histone complexes are devoid of peroxidase activity in contrast to the Hp/Hb complex. Moreover, the biological significance of this inhibition effect of histone on the peroxidase activity of the Hp/Hb complex and the use of its assay as a measure of intravascular hemolysis, is briefly discussed.

Materials and Methods

Preparation of histone

Leukocytes from human blood, and leukocyte nuclei, were isolated by the method previously described (3). The nuclei pellets of human leukocytes were treated with 0.25 mol/l NaCl. The clear supernatant was separated from the nuclei after centrifugation at 2600 *g* for 30 min at + 4 °C. The histones were then precipitated by slow addition of six volumes of cold acetone. The precipitate was prepared by centrifugation, washing 3 times in acetone and ether, and drying under vacuum.

The histones were tested by amino acid analysis as well as by electrophoresis. The protein concentration was checked photometrically.

Preparation of hemoglobin

Human erythrocytes washed five times with saline, were diluted with four parts of water. After careful acidification with 0.1 mol/l HCl to pH 5,8, the erythrocyte membranes were removed by centrifugation. The solution was then neutralized to pH 7.2 with 0.1 mol/l NaOH, centrifuged once again to remove any precipitate, diluted so as to contain exactly 30 g/l of hemoglobin, and stored at – 20 °C (stock solution).

Before use an amount of the stock solution was diluted with saline (600 mg/l hemoglobin).

Materials

Commercial calf-thymus histones, used as control, were obtained from Sigma Chemical Co. (St. Louis, Mo.). Hydrogen peroxide 0.015 mol/l diluted from a stock solution of 300 g/kg (Perhydrol, Merck, Germany); fresh dilutions were prepared each day and standardized by permanganate titration. All other reagents used were of analytical grade. *o*-Dianisidine reagent was prepared by dissolving 1 g *o*-dianisidine (Merck, Germany), 0.5 g EDTA and 15.6 g (0.2 mol) of $NaH_2PO_4 \cdot 2H_2O$ in one liter of distilled water and adjusting the solution carefully to pH 4,10 with H_3PO_4.

Assay of peroxidase activity

The assay of the peroxidase activity was performed according to l. c. (5) as follows: 0.2 ml of serum of healthy donors was mixed with 1.0 ml of the diluted Hb-solution (600 mg/l Hb) in a small tube, incubated 10 min at 37 °C; then 0.02 ml of the mixture was transfered to a test tube containing 5 ml of *o*-dianisidine reagent.

The assay of histone on the Hp/Hb system was performed as follows: To 0.2 ml of serum was added 1.0 ml of a histone solution (0.6 g/l); after incubation for 10 min at 37 °C 1.0 ml of the diluted Hb-solution (600 mg/l) was added. The peroxidase activity was then determined according to the *o*-dianisidine procedure, as in the measurement of the catalase activity of Hp/Hb.

To study the influence of histone on the peroxidase activity of the Hp/Hb complex, the Hp/Hb mixture was incubated for 10 min, followed by the addition of a histone solution.

Each series of analyses should include a reagent blank (water instead of the serum) and a standard of haptoglobin (a serum sample known to contain an excess haptoglobin). The mixture was incubated for 15 min, followed by the addition of 1.0 ml of 0.015 mol/l H_2O_2 to every tube. Absorbance was measured against the blank at 395 nm (Zeiss, Spektralphotometer, model PM 2 DL).

The peroxidase activity was finally calculated with the aid of a standard curve.

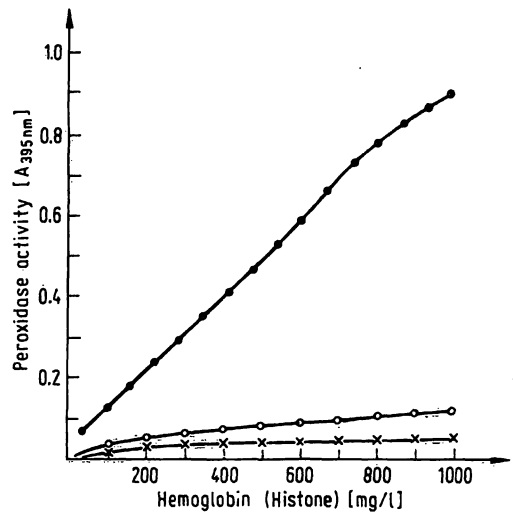

Fig. 1. The peroxidase activity of the complex Hp/Hb (•—•—•), the Hb (o—o—o) and the histone (x—x—x) (for detail see text).

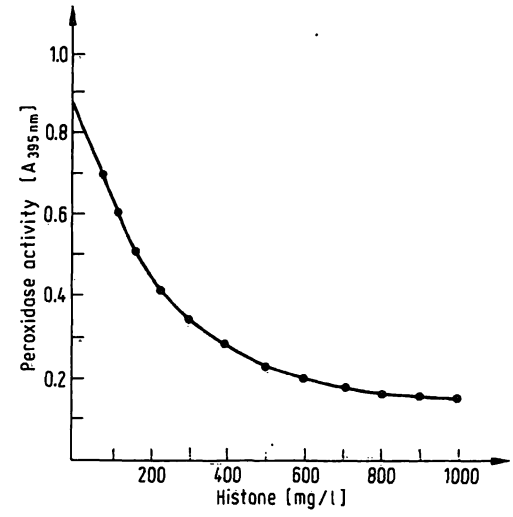

Fig. 2. The inhibition effect of histone on the binding ability of Hb to Hp (in form as reduced peroxidase activity from Hb/Hp complex). Preincubation: Serum + histone, incubation 10 min at 37 °C and then addition of 1,0 ml Hb (diluted Hb-solution). Addition of histone after incubation of Hb-serum hasn't any inhibition effect on the binding of Hp/Hb complex (for detail see text).

Results and Discussion

The Hp/Hb complex possesses classical (1, 2) peroxidase activity, and the level of activity depends on the Hp and Hb concentrations. This is depicted in the graph shown in figure 1. From this it can be seen that the peroxidase activity is directly proportional to the Hb concentrations for the range of 300–800 mg/l Hb, for a standard concentration of Hp (0.2 ml pool serum of 12 healthy individuals).

Human leukocyte nuclear histones show an inhibitory effect on the peroxidase activity of Hp/Hb complexes. This inhibitory effect can be demonstrated using the decrease in peroxidase activity of Hb/Hp complexes in the presence of histones. Figure 2 demonstrates the inhibitory effect of histones on the peroxidase activity of the Hp/Hb complex.

Moreover, it is demonstrated, that the addition of Hb to a preformed Hp-histone complex remains ineffective, namely the interaction of Hp-histone cannot be reversed after the addition of Hb.

The results of these studies indicate, that the human leukocyte nuclear histones inhibit the peroxidase activity of Hp/Hb complexes, and that this phenomenon cannot be reversed. In our laboratory, we found, using immunological methods, an augmentation of the histone concentration in blood plasma during blood storage (9). The interactions between histones, Hb and Hp molecules could be electrostatic, between positively charged and negatively charged molecules. Similar mole-

cular interactions are well known (for review see l. c. (4), (6)). Recently, it was demonstrated (3) that, after a recombination procedure between histones and DNA molecules, according to the concept and method of *Johns & Forester* (7), changes occur in the binding ability of the molecules, probably due to denaturation of the molecules. This alteration is accompanied, of course, by alterations in the biological functions of the corresponding molecules.

However, an interpretation of the selective binding between histones, Hb and Hp, is possible on the basis of molecular interactions between electrostatically different molecules (6).

In conclusion, it appears that human leukocyte nuclear histones can bind to Hp and that the histone-Hp-complexes are devoid of peroxidase activity, in contrast to Hb/Hp complexes. The biological significance of this phenomenon is, that peroxidase activity is not a true measure of intravascular hemolysis, because the leukocyte histones, during cell destruction, can interfere on the formation of Hp/Hb complexes and hinder the binding of Hb to Hp. Thereafter, the free Hb molecules can have a false peroxidase activity. Moreover, it is demonstrated, that the decrease in peroxidase activity of Hp/Hb complexes can be used as a measure for the histone-binding or an assay for the degree of histone-inhibition on the Hp/Hb complex formation.

Conclusion

Structural modifications of cell membranes, as an agglutination of human erythrocytes, are accompanied by intracellular metabolic alterations. The human leukocyte nuclei histones and their subfractions can cause agglutination and/or a hemolysis of human erythrocytes. This phenomenon is specific for the cell membrane receptors of the "ABO blood" group system of the human erythrocytes. The degree of the agglutination and hemolysis depends on the specificity of the erythrocyte membrane receptors, as well as on the structure of histone molecules (different histone fractions) and the protein concentration.

The significance of our results is that the presence of free histones in blood plasma can cause immunological reactions by erythrocytes and probably lymphocytes; with respect to the blood storage and preservation this is of great theoretical and practical interest.

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